Electrogenic arginine transport mediates stimulus–secretion coupling in mouse pancreatic β-cells

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1. We have investigated the mechanism by which L-arginine stimulates membrane depolarization, an increase of intracellular calcium ($[Ca^{2+}]_i$) and insulin secretion in pancreatic β-cells.

2. L-Arginine failed to affect β-cell metabolism, as monitored by NAD(P)H autofluorescence.

3. L-Arginine produced a dose-dependent increase in $[Ca^{2+}]_i$, which was dependent on membrane depolarization and extracellular calcium.

4. The cationic amino acids L-ornithine, L-lysine, L-homoarginine (which is not metabolized) and N^G-monomethyl-L-arginine (L-NMMA, a nitric oxide synthase inhibitor) produced $[Ca^{2+}]_i$ responses similar to that produced by L-arginine. The neutral nitric oxide synthase inhibitors N^G-nitro-L-arginine (L-NNA) and N^ω-monomethyl-L-arginine (L-NAME) also increased $[Ca^{2+}]_i$. D-Arginine was ineffective.

5. L-Arginine did not affect whole-cell Ca^{2+} currents or ATP-sensitive K^+ currents, but produced an inward current that was carried by the amino acid.

6. The reverse transcriptase-polymerase chain reaction demonstrated the presence of messenger RNA for the murine cationic amino acid transporters mCAT2A and mCAT2B within the β-cell.

7. L-Arginine did not affect β-cell exocytosis as assayed by changes in cell capacitance.

8. Our data suggest that L-arginine elevates $[Ca^{2+}]_i$ and stimulates insulin secretion as a consequence of its electrogenic transport into the β-cell. This uptake is mediated by the mCAT2A transporter.

Insulin secretion from pancreatic β-cells is principally controlled by the levels of circulating nutrients. Some of these, such as glucose, act as initiators of release, stimulating secretion in the absence of other agents, whereas others, like arginine, serve as potentiators of release induced by glucose. The mechanism by which glucose stimulates insulin release is now well established (Ashcroft & Rorsman, 1989). At glucose concentrations below ~6 mM, the β-cell is electrically silent, with a resting membrane potential of around −70 mV. This potential is predominantly determined by the activity of ATP-sensitive potassium channels (K_{ATP} channels). An increase in glucose to concentrations > 7 mM stimulates β-cell metabolism. The resulting increase in intracellular ATP, and concomitant fall in ADP, closes K_{ATP} channels and thereby elicits β-cell depolarization and Ca^{2+}-dependent electrical activity. The subsequent rise in $[Ca^{2+}]_i$ stimulates exocytosis of insulin-containing granules.

Arginine is a potent potentiator of insulin release, stimulating secretion in the presence, but not absence, of glucose (Hermans, Schmeer & Henquin, 1987). The mechanism of arginine action is controversial. The most widely favoured hypothesis is that electrogenic uptake of the positively charged amino acid directly depolarizes the β-cell membrane and thus elicits Ca^{2+}-dependent electrical activity, Ca^{2+} entry and insulin secretion (Charles, Tamagawa & Henquin, 1982; Henquin, 1992). Several lines of evidence are in support of this idea. First, the insulinotrophic action of arginine and other cationic amino acids is closely linked to their accumulation in the β-cell (Blachier, Mourtada, Sener & Malaisse, 1989*b*). Second, arginine catabolism is not required since non-metabolizable cationic amino acids are also effective (Blachier *et al.* 1989*a*, *b*). Third, arginine produces β-cell depolarization without causing a reduction in K_{ATP} channel activity (Ashcroft, Ashcroft & Harrison, 1987) or in the resting membrane K^+ permeability (Henquin & Meissner, 1981).

An alternative idea is that arginine evokes insulin secretion by enhancing the production of nitric oxide (NO) within the β-cell. Nitric oxide has been implicated as a major signalling molecule in a wide variety of cell types and is produced

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from arginine by the action of NO synthase (Bredt & Snyder, 1994). The presence of constitutive NO synthase (cNOS) and the production of NO in association with arginine-stimulated insulin secretion has been demonstrated in β-cells (Schmidt, Warner, Ishii, Sheng & Murad, 1992; Bouwens & Kloppel, 1994). Subsequent studies, however, have failed to reproduce these findings (Jones, Persaud, Bjaaland, Pearson & Howell, 1992; Worl, Wiesand, Mayer, Greskotter & Neuhuber, 1994). Furthermore, several studies have shown that high concentrations of cNOS inhibitors stimulate insulin release, rather than inhibit it as would be expected if NO mediates arginine-stimulated insulin secretion (Southern, Schulster & Green, 1990; Jansson & Sandler, 1991; Panagiotidis, Alm & Lundquist, 1992; Panagiotidis, Akesson, Rydell & Lundquist, 1995).

A variant of the NO hypothesis is that cNOS may be activated by the rise in $[Ca^{2+}]_i$ (Bredt & Snyder, 1994) that follows activation of β-cell electrical activity, and that the subsequent elevation of NO potentiates insulin secretion. In favour of this idea is the observation that the sulphonylurea tolbutamide, which depolarizes the β-cell and causes Ca^{2+} entry, can stimulate NO production in β-cells (Schmidt *et al.* 1992). Furthermore, exogenous NO can mobilize intracellular Ca^{2+} stores and cause an increase in $[Ca^{2+}]_i$ in β-cells, which elicits secretion (Willmott, Galione & Smith, 1995). Thus, although it seems unlikely that arginine initiates insulin secretion by a mechanism involving NO production, it remains possible that the amino acid potentiates insulin secretion by enhancing NO synthesis and elevating $[Ca^{2+}]_i$.

To investigate the mechanisms by which arginine induces insulin secretion in β-cells, we have used fluo-3 to monitor changes in $[Ca^{2+}]_i$, the whole-cell patch-clamp technique to investigate effects on membrane currents and the reverse transcriptase-polymerase chain reaction (RT-PCR) to determine which cationic amino acid transporters are expressed in β-cells. Our results suggest that NO production is not involved in the insulinotrophic action of arginine. Rather, the electrogenic uptake of arginine depolarizes the β-cell, elevates $[Ca^{2+}]_i$ and stimulates insulin secretion.

METHODS

Cell isolation

NMRI mice were killed by cervical dislocation and the pancreas removed. Pancreatic islets were then isolated by collagenase digestion and dispersed into single β-cells by low-Ca^{2+} treatment (Duchen, Smith & Ashcroft, 1993). Cells were maintained for up to 4 days in RPMI 1640 tissue culture medium supplemented with 10% fetal calf serum, 10 μU ml^{-1} penicillin and 10 μg ml^{-1} streptomycin at 37 °C in a humidified atmosphere of 5% CO_2 in air. Tissue culture chemicals were obtained from Life Technologies (Paisley, UK).

Fluorescence measurements

NAD(P)H autofluoresence was measured by excitation at 350 nm and emission at 400–500 nm (Duchen *et al.* 1993). Since the spectra of NADH and NADPH overlap it is not possible to distinguish between the signals originating from each source and we therefore refer to them collectively as NAD(P)H. Changes in $[Ca^{2+}]_i$ were monitored using the calcium fluorophore fluo-3, as previously described (Duchen *et al.* 1993). Briefly, cells were loaded with 1 μM fluo-3 AM ester (Molecular Probes) at room temperature (21–23 °C) for 20–30 min in Hanks' solution supplemented with 5–8 mM glucose. The dye was excited at 480 nm and fluorescence monitored at 530 nm. The fluorescence signal was filtered at 1–10 Hz using a 4-pole Bessel filter and stored on DAT pending analysis. Since fluo-3 has a reported K_d for Ca^{2+} of ~1 μM (Molecular Probes) the changes in fluorescence measured are expected to depend linearly on $[Ca^{2+}]_i$ within the physiological range; therefore, the fluorescence signals have not been calibrated in terms of $[Ca^{2+}]_i$ but are presented as percentage changes in signal.

Electrophysiology

Patch pipettes were pulled from borosilicate glass capillaries, coated with Sylgard close to their tips and fire polished immediately before use. They had resistances of 2–5 MΩ when filled with pipette solution.

Membrane potential, whole-cell currents and changes in cell capacitance and conductance were recorded using an EPC-7 patch-clamp amplifier (List Electronic, Darmstadt, Germany). The zero current potential of the pipette was adjusted with the pipette in the bath before establishment of the seal and no corrections have been made for liquid junction potentials. The majority of experiments were carried out using the perforated patch configuration of the patch-clamp method, which retains cell metabolism intact (Smith, Rorsman & Ashcroft, 1990; Duchen *et al.* 1993). The holding potential was −70 mV. Current–voltage (I–V) relationships were obtained by recording the current elicited by a voltage ramp (1–1·5 V s^{-1}) from 0 to −120 mV. Five consecutive ramps were averaged in each solution. The arginine-induced current was obtained by subtraction of the control current from that measured in the presence of the amino acid. Slope conductances were determined by fitting a line to the linear portion of the I–V relationship (between −20 and −100 mV). In some experiments, the standard whole-cell recording configuration was used to measure the effect of L-arginine and NOS inhibitors on whole-cell K_{ATP} currents. Currents flowing through K_{ATP} channels were continuously monitored using alternate ±10 mV, 200 ms pulses applied at a frequency of 0·2 Hz from a holding potential of −70 mV. Data were recorded on DAT and subsequently digitized and analysed using a Digidata A/D converter (Axon Instruments, Burlingame, CA, USA), a 486 computer and in-house software.

Changes in cell capacitance and conductance were measured as previously described (Ämmälä, Eliasson, Bokvist, Larsson, Ashcroft & Rorsman, 1993). Briefly, a 20 mV root mean square 800 Hz sine wave was added to the holding potential (−70 mV) and ten cycles averaged for each data point. The resulting current was analysed at two orthogonal phase angles with a resolution of 100 ms per point. The phase angle was determined empirically for each experiment by varying the G_{series} and C_{slow} knobs on the amplifier. Capacitance changes were evoked by voltage-clamp depolarization.

Solutions

The standard extracellular (Hanks') solution contained (mM): 5·6 KCl, 137 NaCl, 1·2 NaH_2PO_4, 4·2 $NaHCO_3$, 1·2 $MgCl_2$, 2·6 $CaCl_2$, 10 Hepes (pH 7·4 with ~6 mM NaOH) and 0–8·3 mM glucose as indicated. Arginine transporter currents were measured in standard extracellular solution with the following changes: 20 mM NaCl was replaced with 20 mM TEACl to block voltage-gated K^+ currents (final $[Na^+]$, 128 mM); 5·5 mM glucose and 0·5 mM tolbutamide were added to block K_{ATP} currents, and $CaCl_2$ was replaced with $MgCl_2$ (total $[Mg^{2+}]$, 3·8 mM) to block Ca^{2+} currents.

Low-Na^+ solution (11 mM) was made by equimolar substitution of 137 mM NaCl with TEACl. Zero Na^+ solution was made by complete replacement of all Na^+ salts with Cs^+ salts. In standard whole-cell experiments, the bath solution contained (mM): 140 NaCl, 5·6 KCl, 1·2 $MgCl_2$, 2·6 $CaCl_2$ and 10 Hepes (pH 7·4 with NaOH).

For most perforated patch experiments, the pipette solution contained (mM): 70 K_2SO_4, 5 KCl, 5 NaCl, 1 $MgCl_2$, 5 Hepes (pH 7·4 with KOH) and 40 sucrose. For measurement of whole-cell Ca^{2+} currents K_2SO_4 was replaced with Cs_2SO_4. Perforation was produced by inclusion of 0·24 mg ml^{-1} amphotericin B in the pipette solution as previously described (Smith *et al.* 1990; Ämmälä *et al.* 1993) and was considered adequate when the series conductance exceeded 40 nS. For standard whole-cell recordings the pipette was filled with (mM): 107 KCl, 10 NaCl, 2 $MgCl_2$, 1 $CaCl_2$, 10 EGTA, 10 Hepes (pH 7·2 with KOH) and 0·3 K_2ATP.

In all experiments the bath was continuously perfused at 31–33 °C.

Molecular biology

Total RNA was isolated from freshly prepared brain, heart, pancreatic islets and kidney of mouse and also from the mouse insulinoma cell line MIN6 using TRI-Reagent (Molecular Research Centre, Oxford, UK). The total RNA was converted to cDNA by reverse transcription. Two polymerase chain reaction (PCR) primers, sense: 5′-ATGCCTT(CT)GTGGGCTTTGAC-3′ and antisense: 5′-TCAAA(AG)AG(AG)AAGGCCATCAC-3′, were designed to amplify mCAT1, mCAT2A and mCAT2B simultaneously. These were predicted to yield fragments of 421 base pairs (bp) (mCAT1), 419 bp (mCAT2A) and 421 bp (mCAT2B). The PCR conditions consisted of thirty-five cycles at 94 °C for 60 s, 60 °C for 60 s and 72 °C for 60 s. Each 10 μl PCR contained 100 ng of cDNA template, 10 pmol of each PCR primer, 0·2 mM of each of the dNTPs (including 55·5 kBq of [^{32}P]dATP), 0·25 units Taq DNA polymerase (Promega), 50 mM KCl, 10 mM Tris–HCl (pH 9·0), 0·1% Triton X-100 and 1·5 mM $MgCl_2$. The PCR products were digested with *Nar*I for mCATl (to give 389 and 32 bp), *Eco*RI for mCAT2A (to give 292 and 127 bp) and *Bam*HI for mCAT2B (to give 269 and 152 bp).

Analysis

Data were confirmed as having normal distributions and are presented as mean values ± S.E.M. Statistical significance was evaluated using Student's *t* test.

RESULTS

Figure 1 shows simultaneous recordings of membrane potential and $[Ca^{2+}]_i$ from a single pancreatic β-cell and illustrates that in the presence of a substimulatory glucose concentration L-arginine causes membrane depolarization, electrical activity and elevation of $[Ca^{2+}]_i$. Similar results were observed in nine other cells. L-Arginine did not induce electrical activity or increase $[Ca^{2+}]_i$ in glucose-free solutions (data not shown), consistent with earlier observations on intact islets (Hermans *et al.* 1987).

Many nutrient secretagogues mediate β-cell depolarization by stimulating β-cell metabolism. β-Cell metabolism may be monitored by changes in the autofluorescence of the endogenous reducing equivalents NADH and NADPH (Duchen *et al.* 1993). Earlier studies on intact islets of

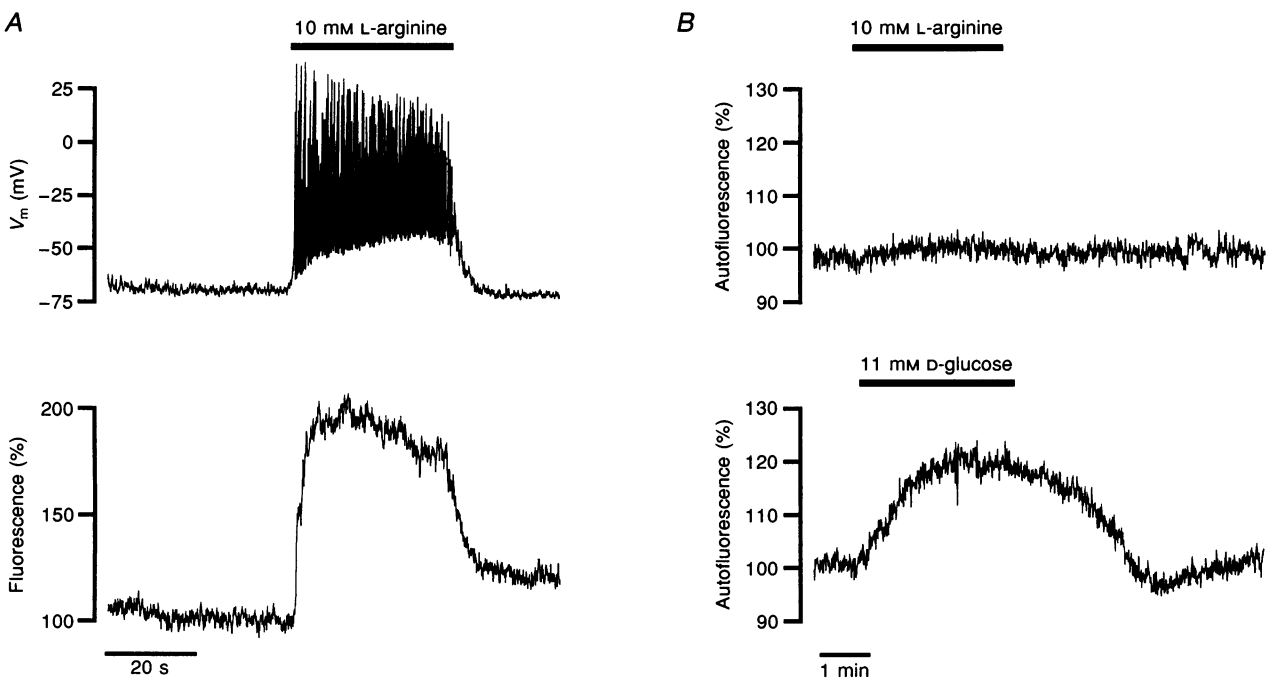

Figure 1. Arginine evokes β-cell depolarization, electrical activity and elevation of $[Ca^{2+}]_i$ without affecting β-cell metabolism

A, simultaneous recordings of membrane potential (top) and $[Ca^{2+}]_i$ (bottom) from a single β-cell. The extracellular solution contained 5·5 mM glucose throughout and 10 mM L-arginine was applied as indicated by the bar. The results are representative of 9 cells. *B*, effects of 10 mM L-arginine (top; applied in the continuous presence of 5·5 mM glucose) or 11 mM glucose (bottom) on NAD(P)H autofluoresence. Data were recorded from the same single cell and are representative of 25 cells.

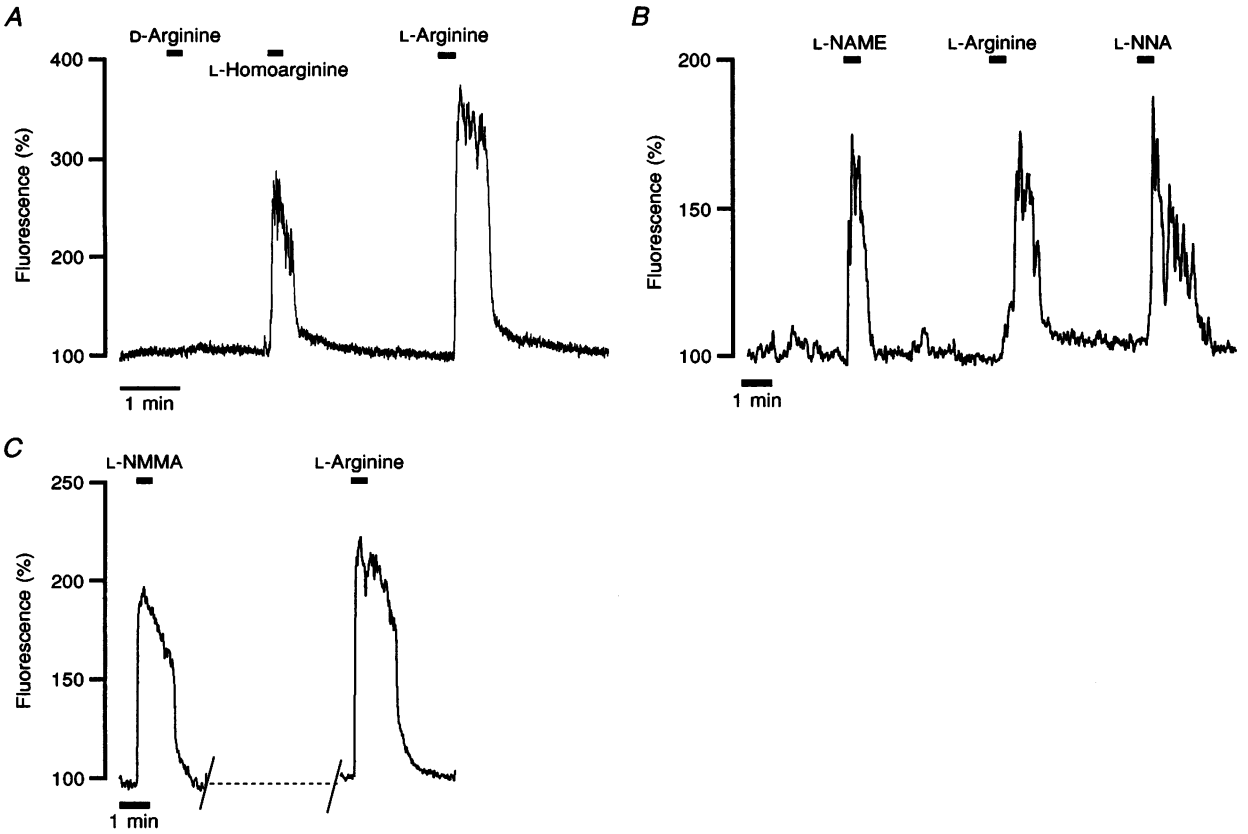

Figure 2. Effects of arginine analogues on $[Ca^{2+}]_i$

A, changes in $[Ca^{2+}]_i$ evoked by 10 mM D-arginine, 10 mM L-homoarginine or 10 mM L-arginine. The amino acids were applied as indicated by the bars; 7 mM glucose was present throughout. The results are representative of 4 cells (D-arginine; L-homoarginine) and 58 cells (L-arginine). *B*, effects of 10 mM L-NAME, 10 mM L-arginine or 10 mM L-NNA on $[Ca^{2+}]_i$, recorded in the presence of 5·5 mM glucose. This was the largest response to L-NNA observed in 8 cells. *C*, effects of 10 mM L-NMMA and 10 mM L-arginine on $[Ca^{2+}]_i$, recorded in the continuous presence of 5·5 mM glucose. The recordings were obtained from the same cell, with an interval of ~7 min. The result is representative of 5 cells.

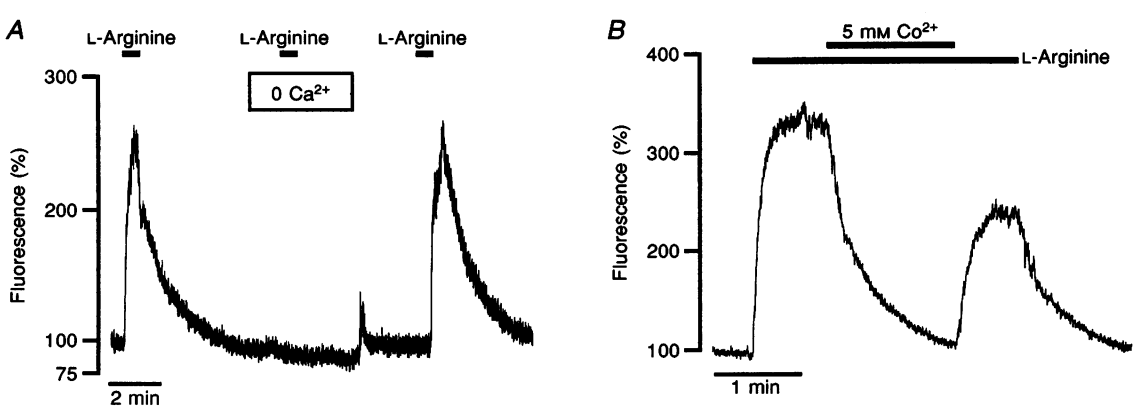

Figure 3. Ca^{2+} influx mediates the rise in $[Ca^{2+}]_i$ evoked by arginine

A, effect of Ca^{2+}-free solution on the $[Ca^{2+}]_i$ transient evoked by 10 mM L-arginine; 7 mM glucose was present throughout. The nominally Ca^{2+}-free solution (< 20 μM) was made by equimolar replacement of $CaCl_2$ with 2·6 mM $MgCl_2$ (total Mg^{2+}, 3·8 mM). Arginine was applied as indicated by the bars. The result is representative of 3 cells. *B*, effect of 5 mM Co^{2+} on the $[Ca^{2+}]_i$ transient elicited by 10 mM L-arginine. Arginine was present during the period indicated by the lower bar. Co^{2+} was added directly to the bath solution in the continued presence of Ca^{2+} as indicated by the upper bar; 7 mM glucose was present throughout. The result is representative of 8 cells.

Langerhans have shown that L-arginine stimulates insulin secretion without altering NAD(P)H autofluoresence (Panten & Christians, 1973). The interpretation of these studies, however, is complicated by the fact that the pancreatic islet contains several different cell types and only 70–80% of islet cells are β-cells (Pipeleers, 1987). We have therefore examined the effect of arginine on NAD(P)H autofluoresence in *single* β-cells. In contrast to glucose, 10 mM L-arginine did not alter the autofluorescence of endogenous NAD(P)H, either in the presence (Fig. 1*B*, $n = 25$) or in the absence of glucose (not shown; $n = 6$). These results support the view that L-arginine is poorly metabolized by the β-cell and that arginine metabolism is not required for the secretory effect of the amino acid. Further evidence in favour of the latter idea is that 10 mM L-homoarginine, a non-metabolizable arginine analogue, also increased $[Ca^{2+}]_i$ (Fig. 2*A*; $n = 4$). Similar results were found with the *N*-substituted arginine-based blockers of NO synthase, N^{ω}-nitro-L-arginine methyl ester (L-NAME), N^{G}-nitro-L-arginine (L-NNA) and N^{G}-monomethyl-L-arginine (L-NMMA; Bredt & Snyder, 1994), which are not metabolized to NO. L-NAME (10 mM; $n = 8$) and L-NMMA (10 mM; $n = 5$) produced rapid and reversible increases in $[Ca^{2+}]_i$ which were comparable in magnitude to those elicited by L-arginine (Fig. 2*B* and *C*). Although 10 mM L-NNA ($n = 8$) also produced a $[Ca^{2+}]_i$ transient, the amplitude of the response was very variable; the largest response obtained is illustrated in Fig. 2*B*. The ability of the NOS inhibitors to elevate $[Ca^{2+}]_i$ implies that the generation of NO is not involved in the response of the β-cell to cationic amino acids.

We next investigated whether the rise in $[Ca^{2+}]_i$ elicited by arginine is due to Ca^{2+} influx from the extracellular solution or to the mobilization of intracellular Ca^{2+} stores. The increase in $[Ca^{2+}]_i$ evoked by L-arginine was reversibly abolished when external calcium was removed (Fig. 3*A*; $n = 3$) and was also blocked by addition of 5 mM Co^{2+}, a non-specific Ca^{2+} channel inhibitor (Fig. 3*B*; $n = 8$). These results indicate that the rise in $[Ca^{2+}]_i$ elicited by L-arginine is mediated by Ca^{2+} influx. $[Ca^{2+}]_i$ transients were only observed in association with membrane depolarization (Fig. 1), indicating that Ca^{2+} enters the β-cell through voltage-gated Ca^{2+} channels activated by the arginine-induced depolarization.

In addition to L-arginine and L-NMMA, the cationic amino acids L-lysine and L-ornithine also elevated $[Ca^{2+}]_i$ in β-cells (10 mM for both, Fig. 4*A*, $n = 11$); the D-arginine enantiomer was ineffective (Fig. 2*A*, $n = 4$). This selectivity suggests that uptake of the amino acid is mediated by the murine cationic amino acid transporter (mCAT) family, but not by the basic amino acid transporter (BAT) or neutral–basic amino acid transporter (NBAT) families (MacLeod, Finley

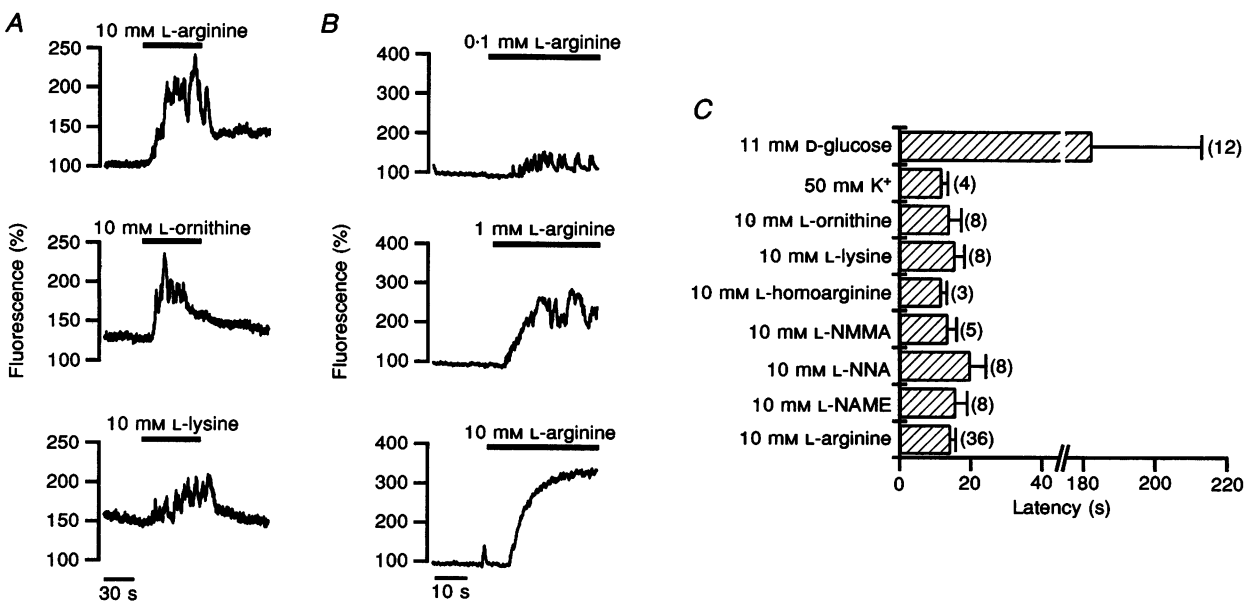

Figure 4. Effects of different cationic amino acids on β-cell $[Ca^{2+}]_i$

A, effects of 10 mM L-arginine, 10 mM L-ornithine and 10 mM L-lysine on $[Ca^{2+}]_i$ in the continuous presence of 8·3 mM glucose. The data come from the same β-cell and are representative of 11 cells. *B*, effect of increasing concentrations of L-arginine on $[Ca^{2+}]_i$; 7 mM glucose was present throughout. The data were recorded successively from the same β-cell and are representative of 5 cells. *C*, mean latency to the start of the $[Ca^{2+}]_i$ transient evoked by: 10 mM L-arginine (14·3 ± 1·5 s, $n = 36$), 10 mM L-NAME (15·8 ± 3·3 s, $n = 8$), 10 mM L-NNA (19·9 ± 4·5 s, $n = 8$), 10 mM L-NMMA (13·6 ± 2·5 s, $n = 5$), 10 mM L-homoarginine (11·7 ± 1·7 s, $n = 3$), 10 mM L-lysine (15·5 ± 2·8 s, $n = 8$), 10 mM L-ornithine (14·0 ± 3·4 s, $n = 8$) and 50 mM K^+ (11·8 ± 1·8 s, $n = 4$). Glucose (5–8 mM) was present throughout each experiment. The top bar shows the mean latency for the $[Ca^{2+}]_i$ transient evoked by an increase in [glucose] from 2·8 to 11 mM (182 ± 31 s, $n = 12$). The number of experiments is given in parentheses. All latencies are significant at the $P < 0·05$ level, with respect to glucose.

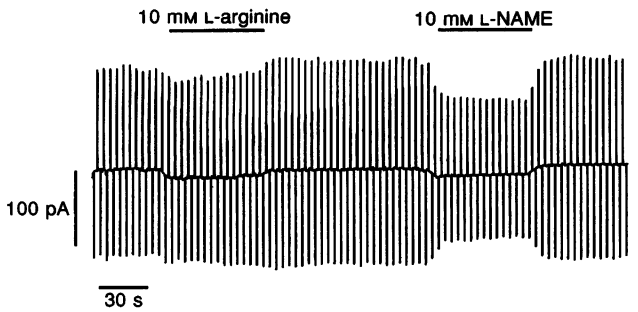

Figure 5. Effect of arginine and L-NAME on the whole-cell K_{ATP} current

K_{ATP} currents recorded from a single β-cell using the standard whole-cell configuration in response to alternate ±10 mV voltage steps from a holding potential of −70 mV.

& Kakuda, 1994; Kakuda & MacLeod, 1994). It also confirms that NO production is not involved in the secretory actions of cationic amino acids, since lysine, ornithine or L-NMMA metabolism did not generate NO. The mean latency for the rise in $[Ca^{2+}]_i$ evoked by basic amino acids was similar to that found with 50 mM KCl and much faster than that measured for 11 mM glucose (Fig. 4*C*). These short latencies suggest that cationic amino acids, like KCl, may elicit rapid Ca^{2+} entry by directly depolarizing the β-cell membrane (Fig. 1*A*).

L-NNA and L-NAME are neutral amino acids that are not expected to be transported by mCAT (Bogle, Moncada, Pearson & Mann, 1992; Schmidt, List, Klatt & Mayer, 1995) but rather via a neutral amino acid uptake system. It is therefore of interest that they both increased $[Ca^{2+}]_i$ with a latency similar to that of L-arginine (Fig. 4*C*). One possible reason for this finding is that L-NAME produces a direct block of the K_{ATP} channel, thereby depolarizing the β-cell and initiating Ca^{2+}-dependent electrical activity (Drews & Krippeit-Drews, 1995; Krippeit-Drews, Welker & Drews,

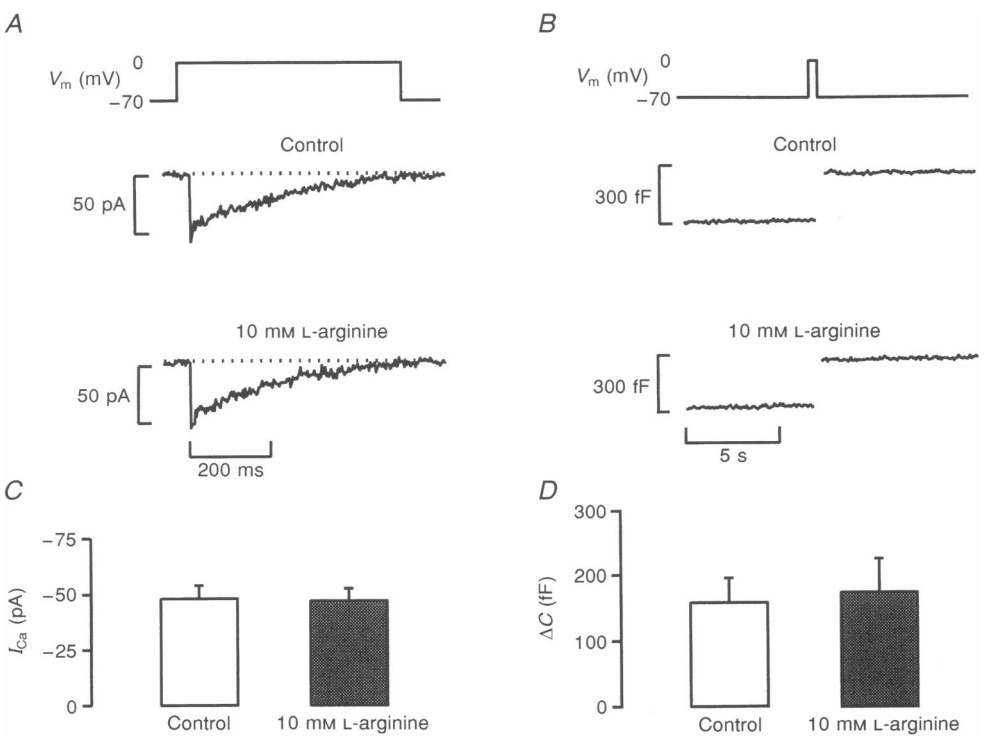

Figure 6. Lack of effect of arginine on Ca^{2+} current and exocytosis in the β-cell

A, inward Ca^{2+} currents recorded from a single β-cell in the absence (middle trace) and presence (lower trace) of 10 mM L-arginine in the bath. Currents were recorded with the perforated patch whole-cell configuration in response to a voltage step (top trace) from a holding potential of −70 mV to 0 mV for 500 ms. *B*, changes in cell capacitance associated with the inward currents shown in *A*. Note the compressed time scale. Upper trace, applied voltage protocol. Middle trace, change in cell capacitance in control solution. Lower trace, change in cell capacitance in the presence of 10 mM L-arginine. *C*, mean peak Ca^{2+} currents in control solution (−48 ± 6 pA, $n = 6$) and in the presence of 10 mM L-arginine (−47 ± 6 pA, $n = 6$). *D*, mean change in cell capacitance before (158 ± 37 fF, $n = 6$) and after (174 ± 50 fF, $n = 6$) application of 10 mM L-arginine.

1996). We therefore investigated the effect of L-NNA and L-NAME on whole-cell K_{ATP} currents. Figure 5 shows that 10 mM L-NAME caused a rapid and reversible inhibition ($30 \pm 3\%$, $n = 7$) of the whole-cell K_{ATP} current. In contrast, 10 mM L-arginine (Fig. 5), 10 mM L-NMMA or 10 mM L-NNA had little effect on the K_{ATP} current, the mean block produced being $0 \pm 2\%$ ($n = 11$), $2 \pm 3\%$ ($n = 4$) and $2 \pm 1\%$ ($n = 10$), respectively. This is in agreement with a previous report that 10 mM L-arginine had no effect on single K_{ATP} channel currents in cell-attached patches (Ashcroft *et al.* 1987).

As shown in Fig. 1, arginine induces β-cell depolarization, and thereby activates Ca^{2+} entry and insulin secretion. Theoretically, membrane depolarization may result either from inhibition of an outward current that is active at the resting potential and/or from activation of an inward current. The only outward current active at the resting potential is the K_{ATP} current, which, as discussed above, was unaffected by L-arginine. The amino acid also did not alter the amplitude or time course of the β-cell Ca^{2+} current (Fig. 6*A*). The mean integrated Ca^{2+} currents recorded at 0 mV in the perforated patch configuration were 48 ± 6 pA ($n = 6$) before, and 47 ± 6 pA ($n = 6$) after, bath application of 10 mM L-arginine (Fig. 6*C*).

To determine whether the inward current induced by L-arginine resulted from electrogenic uptake of the amino acid, we recorded whole-cell currents in the perforated patch configuration using solutions designed to inhibit inorganic ionic currents. Under these conditions, L-arginine (10 mM) induced an inward current of $-3{\cdot}3 \pm 0{\cdot}7$ pA ($n = 20$) at −70 mV (Fig. 7). The amplitude of this current decreased with depolarization. We did not observe reversal of the

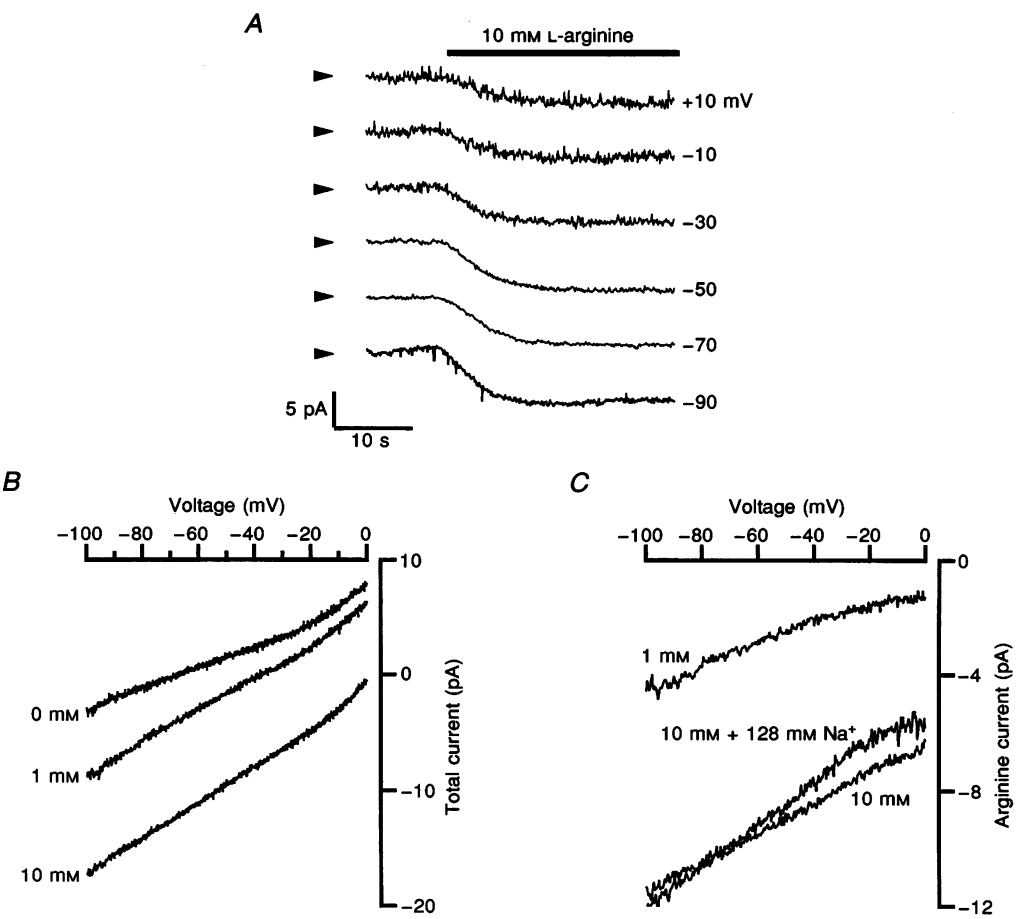

Figure 7. Arginine evokes an inward current in the β-cell

A, whole-cell currents elicited by 10 mM L-arginine (indicated by the bar) at the holding potentials indicated. Arrowheads indicate the preceding level of holding current. Records were obtained consecutively from the same β-cell. *B*, current–voltage relationships recorded from the same β-cell in the absence of arginine, in the presence of 1 mM L-arginine and in the presence of 10 mM arginine. The external solution contained 11 mM Na^+ throughout. *C*, current–voltage relationships for the arginine-induced currents obtained by subtracting the current recorded in the absence of arginine from that recorded in its presence: 1 mM arginine in 11 mM external NaCl (1 mM), the slope conductance was 48 pS; 10 mM arginine in 11 mM NaCl (10 mM), the slope conductance was 89 pS; 10 mM L-arginine in 128 mM external NaCl (10 mM + 128 mM Na^+), the slope conductance was 63 pS.

arginine current at potentials negative to +20 mV. It was difficult to measure arginine currents accurately at more positive potentials because of residual voltage-gated outward currents. The mean slope conductance was 39 ± 10 pS ($n = 20$) with 10 mM L-arginine. When normalized to the cell capacitance, to correct for differences in cell size, the mean current density at −70 mV was −0·5 ± 0·1 pA pF^{-1} ($n = 18$) and the mean slope conductance was 6·1 ± 1·7 pS pF^{-1} ($n = 18$). D-Arginine did not elicit an inward current ($n = 3$, not shown), consistent with its inability to elevate $[Ca^{2+}]_i$. Since K^+ and Cl^- have negative equilibrium potentials under our experimental conditions (−86 and −65 mV, respectively) and external Ca^{2+} was absent, the current activated by L-arginine must be carried either by Na^+ or by the amino acid itself. Reduction of $[Na^+]_o$ from 128 to 11 mM (TEA substitution) did not appreciably alter the slope conductance (Fig. 7*C*). A similar result was observed when external Na^+ was completely replaced with Cs^+: the mean current density and slope conductance were −0·4 ± 0·1 pA pF^{-1} and 3 ± 0·6 pS pF^{-1} ($n = 4$) in the presence and −0·5 ± 0·2 pA pF^{-1} and 3·7 ± 1·2 pS pF^{-1} ($n = 4$) in the absence of external Na^+. We therefore conclude that the current results from the electrogenic transport of the arginine molecule into the β-cell. Arginine currents have not been reported previously in native membranes, but, as discussed below, are observed in *Xenopus* oocytes following expression of cDNA encoding mCAT transporters (Kavanaugh, 1993; Kavanaugh *et al.* 1994).

Arginine currents were also observed in standard whole-cell recordings. The mean amplitude of the current elicited by 10 mM arginine was −8·6 ± 1·2 pA ($n = 11$) at −70 mV, significantly larger than that observed in perforated patch recordings (see above). L-NMMA (10 mM) also elicited an inward current (−5 ± 1 pA, $n = 4$). When normalized to the cell capacitance, the mean current density at −70 mV was −1·6 ± 0·3 pA pF^{-1} ($n = 11$) and −1 ± 0·2 pA pF^{-1} ($n = 4$) for L-arginine and L-NMMA, respectively. No current was evoked by 10 mM L-NNA ($n = 10$).

Our results are consistent with the idea that arginine uptake by β-cells is mediated by a member of the mouse cationic amino acid transporter (mCAT) family. Two mCAT genes

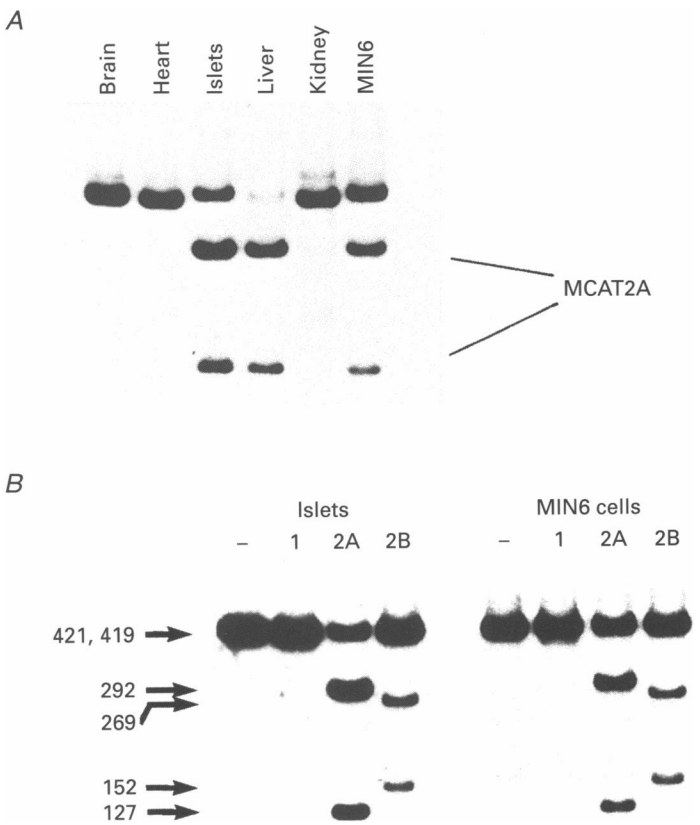

Figure 8. Distribution of mCAT mRNA in β-cells

A, tissue distribution of mCAT2A. The 292 and 127 bp fragments obtained by digestion with *Eco*RI are indicated. *B*, expression of mCAT proteins in islets and MIN6 cells. The bands indicate the restriction patterns obtained by digestion with no enzymes (–), with *Nar*I (1, for mCAT1), with *Eco*RI (2A, for mCAT2A) and with *Bam*HI (2B, for mCAT2B). The size of the restriction fragments (in base pairs) is indicated on the left. Digestion patterns characteristic of mCAT2A and mCAT2B, but not mCAT1, were observed. Since our PCR reaction amplified all three mCAT fragments with similar efficiency (data not shown), an estimate of the relative levels of mRNA can be obtained by comparing the relative densities of the bands obtained in the presence of the relevant restriction enzymes.

have been cloned to date: mCAT1 and mCAT2 (MacLeod *et al.* 1994). The latter is alternatively spliced to give mCAT2A and 2B proteins. mCAT2A has a K_m for L-arginine of 2·7 mM at −60 mV (Kavanaugh *et al.* 1994), whereas mCAT1 and mCAT2B have K_m values of ~0·1 mM and 38 μM, respectively, at −60 mV and are saturated at L-arginine concentrations > 1 mM (Kavanaugh, 1993; Kavanaugh *et al.* 1994). Both the current (Fig. 7*C*) and the $[Ca^{2+}]_i$ transient (Fig. 4*B*) induced by L-arginine were increased when arginine was raised from 1 to 10 mM. Furthermore, uptake of radiolabelled arginine or L-homoarginine by β-cells is half-maximal at 2–3 mM (Hellman, Sehlin & Täljedahl, 1971; Blachier *et al.* 1989*b*). This suggests that arginine uptake into β-cells occurs via mCAT2A. Since mCAT2A mRNA has previously been reported only in the liver (Closs, Albritton, Kim & Cunningham, 1993; MacLeod *et al.* 1994), the tissue distribution of mCAT2A mRNA was re-examined. We found mCAT2A mRNA to be strongly expressed in both mouse islets and in the mouse insulinoma line MIN6, which constitutes a pure β-cell population (Fig. 8*A*). As previously reported (Closs *et al.* 1993), mCAT2A mRNA was not detected in brain, heart and kidney (Fig. 8*A*). In addition to mCAT2A, mCAT2B mRNA was detected in both islets and MIN6 cells (Fig. 8*B*). In islets, ~70% of the mCAT mRNA is mCAT2A, ~30% is mCAT2B and there is almost no mCAT1, while in MIN6 cells mCAT2A constitutes ~50% of the total mCAT mRNA. The differences in relative expression levels between MIN6 cells and islets may reflect the fact that the mouse islet is comprised of approximately 70% β-cells (Pipeleers, 1987).

Many potentiators of insulin secretion not only increase $[Ca^{2+}]_i$ but may also promote insulin secretion by generating second messengers that interact directly with the secretory machinery (Ämmälä *et al.* 1993). This is not the case for arginine, however, as it had no effect on the membrane capacitance changes which reflect exocytosis of secretory granules (Fig. 6*B*). The mean capacitance increase evoked by membrane depolarization to 0 mV was 158 ± 37 pF before, and 174 ± 50 pF after, application of 10 mM arginine ($n = 6$, Fig. 6*D*).

DISCUSSION

Our results support the idea that L-cationic amino acids, such as arginine, lysine and ornithine, stimulate insulin secretion as a consequence of the inward current that results from the transport of the positively charged amino acid into the β-cell by mCAT2A. This current produces a membrane depolarization that stimulates voltage-gated Ca^{2+} entry, a rise in $[Ca^{2+}]_i$ and thereby insulin secretion.

Arginine acts as a potentiator, but not an initiator of insulin secretion; that is, it is only effective in the presence of stimulatory, or just substimulatory, glucose concentrations. A similar effect is found for the action of the amino acid on the electrical activity of the β-cell. Electrophysiological recordings from intact islets have shown that at a substimulatory glucose concentration of 3 mM the β-cell is electrically silent with a membrane potential of −70 mV (Hermans *et al.* 1987). Addition of arginine under such conditions depolarizes the membrane to approximately −60 mV, which is not sufficient to reach the threshold for electrical activity (between −50 and −40 mV). In the presence of 7 mM glucose, however, the β-cell input resistance is higher and the resting potential more depolarized (> −60 mV) so that arginine now depolarizes sufficiently to elicit electrical activity and insulin secretion (Hermans *et al.* 1987). At higher glucose concentrations (> 7 mM) arginine increases the ambient electrical activity, thereby potentiating insulin secretion.

Our data can account for this glucose dependence of the effects of arginine on β-cell electrical activity. In the absence of glucose, the β-cell has a membrane potential of −70 mV and an input resistance of ~0·5 GΩ (Smith *et al.* 1990). Under these conditions, 10 mM arginine will produce an inward current of −3·3 pA but the resulting depolarization (< 2 mV) will be insufficient to elicit electrical activity. When glucose concentration is increased to 5–8 mM, K_{ATP} channels close, the input resistance increases to > 4 GΩ and the β-cell depolarizes to approximately −55 mV (Smith *et al.* 1990). Arginine (10 mM) will now produce an approximately −3 pA inward current and depolarize the β-cell by ~10 mV, sufficient to elicit electrical activity and stimulate insulin secretion. At higher glucose concentrations the inward arginine current will enhance electrical activity and potentiate insulin release. The effect of arginine therefore depends on the input resistance and resting potential of the β-cell. Since both of these parameters are modulated by glucose (via its effect on K_{ATP} channels) the arginine response will vary with the extracellular glucose concentration. The effect of arginine will also depend on the size of its inward current and thus on the level of mCAT2A expression.

Our results therefore explain why arginine acts only in the presence of glucose, being a potentiator but not an initiator of insulin secretion. They also imply that electrogenic arginine uptake via mCAT2A is unlikely to depolarize hepatocytes, which have an input resistance of ~0·2 GΩ (Field & Jenkinson, 1987); in order to depolarize a hepatocyte by 10 mV an estimated arginine-associated current in excess of 50 pA would be required.

If we assume the transport of a single arginine molecule corresponds to the transfer of one net positive charge across the β-cell membrane (i.e. there is no cotransport of anions or countertransport of cations), the current produced by 10 mM arginine at −70 mV will result from an influx of 10^9 arginine molecules per minute. In a β-cell with a volume of approximately 1 pl this corresponds to an accumulation of intracellular arginine of ~2 mM min^{-1}. This argues that, in the absence of mechanisms to remove arginine, its intracellular concentration will reach 10 mM within 6 min. The resulting decrease in its concentration gradient may be expected to reduce uptake of the amino acid. L-Homoarginine is a non-metabolizable substrate for mCAT2A with

a similar affinity to arginine (2–3 mM; Bogle *et al.* 1992; Schmidt *et al.* 1995). Uptake studies in intact islets incubated in 10 mM radiolabelled L-homoarginine, also suggest that the intracellular concentration reaches > 10 mM within 3 min (Blachier *et al.* 1989*b*). Intracellular accumulation of arginine may help explain why in some intact β-cells we observed a progressive decline in both the arginine current and the arginine-evoked $[Ca^{2+}]_i$ response with time. The finding that arginine currents measured in the standard whole-cell recording configuration did not noticeably decline and were larger than those measured using the perforated patch configuration, also supports this argument. This is because in standard whole-cell recordings arginine entering the cell does not accumulate but dialyses with the much larger volume of pipette solution.

L-NAME, L-NNA and L-NMMA have all been used to investigate the role of cNOS in both normal β-cell function and arginine stimulation of insulin secretion (Jansson & Sandler, 1991; Jones *et al.* 1992; Panagiotidis *et al.* 1992, 1995). In the presence of substimulatory glucose concentrations, they all potentiate insulin release. Our observation that these compounds, albeit at concentrations in excess of those required to block cNOS, themselves increase $[Ca^{2+}]_i$ explains why they stimulate insulin secretion. Like L-arginine, L-NMMA generates an electrogenic inward current and thereby elicits β-cell depolarization, elevation of $[Ca^{2+}]_i$ and insulin secretion. In contrast, L-NAME stimulates insulin release by directly blocking the β-cell K_{ATP} channel (Fig. 5; Krippeit-Drews *et al.* 1996). Our results do not suggest a mechanism by which the neutral amino acid L-NNA increases $[Ca^{2+}]_i$.

The finding that L-NMMA, L-ornithine and L-lysine, none of which are metabolized to NO, were effective at elevating $[Ca^{2+}]_i$ argues against the idea that arginine evokes insulin release by activation of NO synthase. It seems possible, therefore, that the ability of secretagogues to enhance NO release from insulinoma cells (Schmidt *et al.* 1992) is a consequence, rather than the cause, of depolarization-mediated Ca^{2+} entry.

Our results suggest that both mCAT2A and mCAT2B are strongly expressed in β-cells whereas mCAT1 is expressed at much lower levels, if at all. Since the fasting plasma concentration of cationic amino acids (arginine, ornithine and lysine) is ~0·6 mM (Toback, Nayers & Lowenstein, 1973), mCAT2B is likely to be saturated under resting conditions. mCAT2B may therefore be considered as a house-keeping gene. The mCAT2B gene is induced in other tissues that, like the β-cell, express inducible nitric oxide synthase (iNOS; Southern *et al.* 1990; MacLeod *et al.* 1994). Our detection of mCAT2B in freshly isolated islets argues that it is constitutively present in β-cells. In contrast to mCAT2B, which is already saturated at resting plasma amino acid concentrations, uptake via mCAT2A will be increased by the elevated amino acid levels (> 1 mM; Toback *et al.* 1973) that occur after a protein meal. It is of interest that the liver and the pancreatic β-cell share the same isoform of the mCAT2 transporter. A similar finding has also been reported for several other enzymes (e.g. glucokinase) and may reflect the fact that the β-cell and the liver both play important roles in the regulation of plasma nutrient levels.

Arginine is often used in clinical studies to assess the insulin secretory capacity of normal and diabetic patients. In such studies, the insulin response to an intravenous bolus of arginine is measured. In non-insulin-dependent diabetes mellitus (NIDDM) the β-cell secretory response to glucose is reduced. A similar decrease in the secretory response to arginine is also observed, even when the arginine response in normal and diabetic subjects is measured at an identical glucose concentration (Ward, Bolgiano, McKnight, Halter & Porte, 1984; Ward, Beard, Halter & Porte, 1985; Dimitriadis, Pehling & Gerich, 1985). Such data have led to the suggestion that NIDDM may be associated with a secretory defect in the β-cell that occurs distal to K_{ATP} channel closure. We point out, however, that the effect of arginine is contingent on both the input resistance and the membrane potential of the β-cell, and thus the level of K_{ATP} channel activity. The reduced insulin secretory response to arginine observed in NIDDM patients may therefore simply be secondary to a defect in the K_{ATP} channel. An alternative explanation is that it results from diminished β-cell expression of mCAT2A, resulting in a smaller inward arginine current. The failure of arginine to stimulate insulin secretion in NIDDM may therefore involve a defect in membrane-mediated events, rather than processes distal to β-cell depolarization.

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